FULL METHODS

ATR activation

To generate the 800-bp telomeric dsDNA fragment, the pSTY11 plasmid (a gift from Dr. Titia de Lange, Rockefeller University) was digested with EcoRI and the excised fragment was gel purified. The 800-bp random sequence dsDNA was generated by PCR and column purified. These dsDNA fragments were incubated with T7 exonuclease for 15s at room temperature and flash frozen in an ethanol-dry ice bath. T7 was inactivated by subsequent incubation at 70°C for 20 minutes and DNA fragments were separated on 2% agarose gel to confirm equal resection. The resected DNA fragments were incubated with NE as previously described[14]. To specifically monitor the phosphorylation of RPA2 by ATR and eliminate the contributions of ATM and DNA-PK to RPA2 phosphorylation, NE were pretreated with 20 μM KU55933 and NU7026 inhibitors for 15 minutes at 4°C. The extracts were mixed with the DNA fragments, incubated for 15 minutes at 37°C, and RPA phosphorylation was analyzed by Western blot.

Protein purification

The POT1-TPP1 complex was either purified from baculovirus-infected Sf9 cells as previously described[16], or purified from HEK293E cells as follows: The pCL-Flag-POT1 and pCL-Flag-TPP1 vectors[16] were individually transfected or cotransfected into HEK 293E cells. The cells were collected after 72 hours and lysed in the NETN buffer [100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% NP-40, and protease inhibitors], sonicated, and cleared by centrifugation (10,000 x g for 10 min). The cleared lysates were incubated with the M2 anti-Flag antibody-conjugated beads at 4°C for 2 hours and eluted with 200 μg/ml 3X Flag peptide in Buffer A [25 mM Tris-HCl (pH 8.0) 100 mM NaCl,

10% glycerol] for 1 hour. Recombinant RPA complex was purified from *E. coli* as previously described[30]. hnRNPA1 pET9d plasmid (a gift from Dr. Adrian Krainer, Cold Spring Harbor Laboratory) was transformed into *E. coli* and expression was induced with IPTG (0.4 mM) for 3 hrs at 37°c. The cells were then collected and lysed in binding buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 μg/ml BSA, 10% glycerol, 0.05% NP-40]. Lysates were sonicated, cleared by centrifugation (10,000 x g for 10 min), and incubated with ssTEL (50 μM) conjugated M280 beads (100 μl) for 30 minutes at room temperature. The ssTEL and associated protein were captured by magnets, washed in binding buffer, and eluted with 1 M NaCl for 10 minutes at 4°c. The eluted protein was then diluted in binding buffer without salt to bring the final NaCl concentration down to 100 mM. Single-stranded binding protein (SSB) was purchased from Promega.

Gel-shift assay

The 18-nt telomeric ssDNA probe [$(TTAGGG)_3$] was radiolabled with γ–^{32}P using T4 kinase and purified over a G25 column. The labeled ssDNA was incubated with purified RPA or POT1-TPP1 in binding buffer [10mM Tris-HCl (pH 7.5), 100mM NaCl, 10 μg/ml BSA, 10% glycerol, 0.05% NP-40] for 30 min at room temperature. The resulting protein-DNA complexes were separated by gel electrophoresis using 0.8% agarose at 140 volts for 1.5 hr and bands were visualized by autoradiography.

DNA-protein binding assay using biotinylated ssDNA

Biotinylated ssTEL [$(TTAGGG)_8$] or ssMUT [$(TTTGCG)_8$] were attached to streptavidin-coated magnetic beads in 10 mM Tris-HCl (pH 8.0), 100 mM NaCl at room temperature for 30 min. For analyzing the bindings of purified RPA, POT1-TPP1, and POT1 to

ssDNA, biotinylated ssDNA (1 pmol) was incubated with various amounts of purified protein in 500 μl of binding buffer. For analyzing the binding of RPA and Flag-POT1 to ssDNA in extracts, biotinylated ssDNA (10 pmol) and various amounts of extracts were added to 500 μl of binding buffer. Following a 30-min incubation, the protein-DNA complexes were retrieved with a magnet and washed 3 times with binding buffer. In the experiments using RPA or POT1 pre-coated ssDNA, biotinylated ssDNA (1 pmol) was first incubated with purified protein (3.8 pmol) for 30 min at room temperature. The ssDNA pre-coated with RPA or POT1 was retrieved with a magnet and subsequently mixed with increasing concentrations of WCE, NE, or CE for 30 min at room temperature. For NE inhibited by addition of TERRA or its derivatives [$(UUAGGG)_3$, $(CCCAUU)_3$, and $(UUGGCG)_3$], extracts were incubated with 1, 2, 5, or 10 pmol RNA for 30 minutes at 4°C.

For hnRNPA1 binding, RPA-coated ssTEL or ssMUT (0.8 nM), or Flag-POT1-coated ssTEL (0.8 nM), were incubated with increasing concentrations of hnRNPA1 purified from *E. coli* (2.4, 4.8, 7.2 nM) and the proteins remaining on ssTEL were analyzed by Western blot. For TERRA inhibition, hnRNPA1 was preincubated with increasing concentrations of TERRA (2, 4, 10, 20 nM), or control RNA $(UUGGCG)_3$. hnRNPA1 was then incubated with RPA coated ssTEL (0.8 nM). Similarly, to demonstrate that TERRA promotes the dissociation of hnRNPA1 from ssTEL, the ssTEL (0.8 nM) was precoated with hnRNPA1 (2.4 nM) and subsequently incubated with increasing concentrations of TERRA (2, 20, 200, 2000 nM). To demonstrate that TERRA enhances POT1 binding, ssTEL (0.8 nM) was precoated with hnRNPA1 (2.4 nM) and then incubated with both POT1 (2.4 nM) and increasing concentrations of TERRA (2, 20, 200 nM). In all reactions, the proteins remaining on DNA were analyzed by Western blot.

Cell synchronization

To follow the progression of cells from S to G2 (Fig. 4b, 4e), HeLa cells were synchronized with 2 mM thymidine for 16 hrs, washed three times with PBS and once with thymidine-free medium, and released into thymidine-free medium. To enrich HeLa cells in S phase of the cell cycle (Fig. 4a), cells were either collected after a 16-hr treatment with 2mM thymidine (early S), or collected 4 hr after thymidine release (late S). To enrich cells in G1 and M phases, cells were either collected after a 16-hr treatment with 0.1 μg/ml nocodazole (M), or collected 4 hr after nocodazole release (G1).

Extract preparation

WCEs were either generated with the NETN buffer as described in the protein purification section, or with the binding buffer used in the DNA binding assays. NE and CE were generated as previously described[3]. To treat extracts with TERRA or its derivative RNA, RNA were added to WCE or NE in increasing concentrations (1, 2, 5, 10 pmol) and incubated for 30 minutes on ice.

Capture of RPA displacing activity from extracts

To capture the RPA displacing activity from extracts, RPA-coated ssTEL was incubated with NE for 30 minutes at room temperature. The beads were collected, washed 3 times in binding buffer, and eluted using the binding buffer with 1 M NaCl for 10 minutes on ice. The eluted material was collected, and diluted with the binding buffer without NaCl to reach a final NaCl concentration of 100 mM. The elution was incubated on ice for 1 hour and then added to RPA-coated ssDNA and incubated for 30 minutes at room temperature. For TERRA inhibition, either TERRA $(UUAGGG)_3$ or its derivative $(CCCAUU)_3$ were

incubated with the eluted proteins prior to their addition to RPA-coated ssDNA. The proteins remaining bound to DNA were analyzed by Western Blot.

Identification of the RPA displacing factors from extracts

Biotinylated ssTEL or ssMUT (20 pmol) was attached to streptavidin-coated beads and coated with recombinant RPA. The RPA-coated ssTEL or ssMUT was incubated with 65 μg of NE in 500 μl of binding buffer. Beads with no DNA attached were used a negative control. After 30 min of incubation, the beads were retrieved and washed 3 times with binding buffer containing 300 mM NaCl. The proteins associated with the RPA activity were eluted by binding buffer containing 600 mM NaCl for 10 minutes on ice. The eluted proteins from ssTEL, ssMUT, and naked beads were separated on a SDS PAGE. After the gel was silver-stained, the two ~30 kDa bands specifically captured by RPA-ssTEL were excised and analyzed by mass spectrometry.

Immunofluorescence analysis

HeLa cells were seeded onto coverslips and cultured overnight. The adhered cells were transfected with POT1 siRNA using oligofectamine (Invitrogen), or with hnRNPA1 siRNA using Lipofectamine RNAi Max (Invitrogen) and cultured for another 48 hrs. Synchronized cells were treated after 24hrs with 2 mM thymidine for 16 hr, washed and released, and processed at the indicated time points. Cells were extracted with 0.25% Triton, fixed in 3% paraformaldehyde, and further permeablized with 0.5% Triton. Cells were subsequently incubated with the primary antibodies (diluted in PBS containing 3% BSA and 0.05% Tween 20) for 1 hr at 37°C in a humidified chamber. Following extensive washing with PBS, cells were incubated with secondary antibodies for 45 min at room

temperature, and washed again with PBS. After a 5-min incubation with DAPI, cells were mounted on slides with Vectashield. Slides were analyzed using a Nikon H600L fluorescence microscope.

Combined Immunofluorescence-RNA FISH

Cells were grown on coverslips and collected at different time points 17 h after release of single thymidine block. Cells were washed twice with cold PBS for 5 min and treated with cytobuffer (100mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM PIPES pH 7, 0.1% Triton X-100, 200mM Vanadyl Ribonucleoside Complex) for 7 min at 4°C. Cells were rinsed briefly, fixed with 4% paraformaldehyde in PBS (USB 19943) for 10 min at RT. Cells were then washed three times with PBS for 5 min each and permeabilized with 0.5% NP40 in PBS for 10 min. Cells were washed twice with PBS for 5 min each and incubated with blocking solution (0.2% Fish Gelatin and 0.5% BSA) for 1 hour. Cells were then incubated with human TRF2 antibody (clone 4A794 Upstate) at 1:2000 diluted in blocking solution for 2 h. After washing three times with PBST (PBS containing 0.1% Triton) for 10 min each, the cells were than incubated with secondary antibody Alexa 488 (Invitrogen A11001) at 1:2000 dilution in blocking solution for 1 h. Cells were washed three times with PBST for 10 min each were fixed with 4% paraformaldehyde in PBS for 10 minutes at RT. Cells were rinsed briefly with PBS and then incubated with hybridization mix (10 nM PNA-TAMRA-(CCCTAA) probe, 50% formamide, 2x SSC, 2mg/ml BSA, 10% dextran sulfate, 10mM Vanadyl Ribonucleoside complex) for 18 hours in a humidified chamber at 39°C. Cells were washed with 2x SSC in 50% formamide three times at 39°C for 5 min each, three times in 2xSSC at 39°C for 5 min each, and finally one time in 2x SSC at RT for 10 min. Coverslips were than mounted on glass microscope slides with

vectashield mounting medium containing DAPI (H-1200). For RNaseA treatment, coverslips were incubated with 200 μg/ml RNase A for 30 min at 37 ^{o}C prior to hybridization. Images were captured with an Endore cooled CCD camera on a Nikon eclipse 80*i* microscope and the images were processed with NIS-Element BR 3.10 software.

Chromatin immunoprecipitation (ChIP)

RPA ChIP and the analysis of telomere association were performed as previously described[7]. Cells were transfected twice with hnRNPA1 siRNA (hnRNPA1-1) and synchronized with thymidine for 15 hr. The two RPA2 antibodies used are from Abcam and Thermo.

Antibodies and siRNA

The RPA pS33 antibody is from Bethyl. The monoclonal antibody to RPA2 is from Neomarkers. The anti-FLAG M2 antibody is from Sigma. The Chk1 antibody and Cyclin A antibody are from Santa Cruz, and the phospho-Chk1 Ser345 antibody is from Cell Signaling. The TRF2 antibody is from Bethyl. The phospho-H3 Ser10 antibody is from Millipore. The H4 antibody is from Active Motif. The hnRNPA1 antibody is from Cell Signaling. The POT1 siRNA used in Fig 4e and S10 is the SMARTPOOL from Dharmacon. The hnRNPA1 siRNAs used in Fig 4c-d and S8-9 are CAACUUCGGUC-GUGGAGGA and UCCACGACCACCACCAAAG.

Reference:

30. Henricksen, L. A. & Wold, M. S. Replication protein A mutants lacking phosphorylation sites for p34cdc2 kinase support DNA replication. J Biol Chem 269, 24203-8 (1994).